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Dear Miss Morgan:

Enclosed are three copies of an article entitled
"Chlorpromazine Metabolism in Sheep II. In Vitro
Metabolism and Preparation of ³H-7-Hydroxychlorpromazine"
by L. G. Brookes, M. A. Holmes, I. S. Forrest,
V. A. Bacon, A. M. Duffield and M. D. Solomon.
This research was supported in part by NASA Grant
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Very truly yours,

Muriel Allan
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CHLORPROMAZINE METABOLISM IN SHEEP

II. IN VITRO METABOLISM AND FRACTIONATION OF ^3H -7-HYDROXYCHLORPROMAZINE

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Sheep liver microsomes incubated with chlorpromazine produced the normal spread of known metabolites of the substrate drug usually seen in vitro. They showed an outstanding capacity for 7-hydroxylation. The fresh microsomal preparations converted from one-third to more than two-thirds of the substrate into various 7-hydroxylated derivatives of chlorpromazine. Manipulation of storage conditions of the microsomal fractions prior to incubation with drug substrate furthermore resulted in the production of nearly 70% of 7-hydroxychlorpromazine, at the expense of other non-phenolic and phenolic metabolites. Thus, in sheep liver microsomes the pathways for 7-hydroxylation were found to be more resistant to deterioration in storage than those for demethylation, N-oxidation and sulfoxidation.

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An inter-species study of in vitro ^3H -chlorpromazine ($^3\text{H-CP}^*$) metabolism was carried out, using hepatic microsomes of 13 species of mammals (Brookes, Forrest and Hubbard, 1970; Brookes and Forrest, 1971). It was found that some of the species showed the propensity of producing large percentual amounts of specific metabolites. Thus, guinea pig and dog hepatic microsomes produced more than 60% chlorpromazine-N-oxide (CPNO), Rhesus monkey and rat yielded in excess of 20% nor_1 -chlorpromazine (nor_1CP), rabbit was the only species to yield substantial amounts (approximately 15%) of nor_2 -chlorpromazine (nor_2CP), while sheep produced more than 30% 7-hydroxy-chlorpromazine (7-OHCP).

Since 7-OHCP and its derivatives are important biotransformation products of CP in man and most mammals, either unconjugated or as the aglycones of glucuronic acid or other conjugates (Beckett, Beaven and Robinson, 1963; Goldenberg and Fishman, 1965; Bolt and Forrest, 1967; Forrest, Kosek et al., 1970), it was previously synthesized as reference compound for

• Abbreviations used:

CP	= chlorpromazine
nor_1CP	= desmonomethylchlorpromazine
nor_2CP	= desdimethylchlorpromazine
CPNO	= chlorpromazine-N-oxide
CPONO	= chlorpromazine sulfoxide-N-oxide
CPO	= chlorpromazine sulfoxide
nor_1CPO	= desmonomethylchlorpromazine sulfoxide
7-OHCP	= 7-hydroxychlorpromazine
$\text{nor}_1\text{7-OHCP}$	= desmonomethyl-7-hydroxychlorpromazine
7-OHCPO	= 7-hydroxychlorpromazine sulfoxide
$\text{nor}_1\text{7-OHCPO}$	= desmonomethyl-7-hydroxychlorpromazine sulfoxide
8-OHCP	= 8-hydroxychlorpromazine

studies of CP metabolism by laborious, multi-step procedures (Nodiff and Hausman, 1966; Nodiff, Ina et al., 1967). When it was noted that sheep liver microsomes exceeded those of all other species in their capacity for 7-hydroxylation of CP by a wide margin, it was attempted to try this route as an alternate method of preparing ^3H - or unlabeled 7-OHCP (Forrest, Brookes and Barth, 1970).

MATERIALS AND METHODS

Sheep livers were obtained upon sacrifice from a local slaughterhouse, transported to the laboratory under ice cooling, and processed immediately. 100 g of the liver specimens were homogenized with ice-cold isotonic KCl (1.15% w/v) in a Waring blender and centrifuged at -5°C for 30 minutes at 9000 G. The supernatant served as the source of microsomes. For incubation of the microsomal fraction with the substrate CP, the equivalent of 4 g of liver (12 ml) was used, to which were added the co-factors MgCl_2 (40 μmol), nicotinamide (240 μmol), glucose-6-phosphate (48 μmol), NADP (8 μmol), and ATP (1.4 μmol). The substrate used was 8 μmol CP.HCl, which contained the equivalent of 20 μCi of tritiated CP*. The incubation mixture was made up with 14 ml Sørensen's phosphate buffer pH 7.4, to a total of approximately 32 ml, and incubation was carried out in air at 37°C in a shaking incubator for 50 minutes, the optimal time period previously determined (Brookes, Forrest and Hubbard, 1970). The mixture was then exhaustively extracted with dichloromethane (3 x 160 ml) at pH 9.2. The pooled organic phases were taken to dryness on a rotary evaporator, and the residue taken up in 0.5 ml ethyl acetate. Aliquots of the ethyl acetate

* labeled at position 9 of the nucleus

phase were used for direct scintillation counting and also for spotting on silica gel H (Merck) plates, coated with either 250 μ m or 1 mm layers of support, for analytical or preparative purposes, respectively.

³H-Chlorpromazine Metabolites Formed In Vitro

For analytical purposes, 5 to 10 μ l, i.e. 1% to 2% of the ethyl acetate phase, were spotted on a thin layer plate, which was developed in the solvent system butanol - absolute ethanol - diethylamine, (12:6:1) (Forrest, Bolt and Serra, 1968), sprayed with sulfuric acid - water - ethanol (95%), (1:1:6). All sulfides and phenolic derivatives were demonstrated immediately, as pink or purple spots, while non-phenolic sulfoxide spots were visualized as darker pink spots by gentle heating with a hot air drier. All spots were then scraped off the silica gel, placed in scintillation vials, incubated for 30 minutes at 60°C with 5 ml of a composite scintillation fluid, containing PPO, H₂POPOP and toluene (5 g PPO, 0.25 g H₂POPOP, made up to 1000 ml with toluene). A Packard Tri-Carb Scintillation Counter was used for radio-quantitation.

RESULTS AND DISCUSSION

The results of various microsomal incubations are summarized in Table I. The first two vertical columns I and II represent pools of two livers each from animals of undetermined breed and sex. Columns III to VI represent single livers, from 3 males and 1 female, and column VII represents the pool of microsomes from livers III to VI. The four livers represented in columns I and II, and III to VI, respectively, were obtained on different days. The animals reaching the slaughterhouse were 5 to 10 months old, according to the veterinary inspector. There may have been a difference of age and/or breed accounting for the differences seen in total number of metabolites. The single livers, III to VI, may have come from older animals which would

explain the larger number of metabolites formed, and the greater number of hydroxylated and sulfoxidated metabolites formed in these instances. However, a strain difference can not be excluded (Brookes, Holmes et al., 1970). The differences in total metabolic potential, leaving 30% to 40% of applied CP unmetabolized in the tests of columns I and II, while only 1% to 2% remained unmetabolized by the microsomes of the individual livers III to VI, would be in keeping with age as the determining factor, since older, i.e. fully mature animals of other species showed similar differences within the same strain, both in vivo and in vitro (Brookes, Holmes et al., 1970). The fact that the lambs (preceding paper, Tables I-III) showed a mature type of CP metabolism in vivo was probably due to previous induction of drug-metabolizing hepatic enzyme systems, and does not argue against age as the cause of differences observed in the livers of naive animals.

Co-factor requirements were studied only with regard to the yield of 7-OHCP (Forrest, Brookes and Barth, 1970). It was found that $MgCl_2$ could be omitted without any noticeable effect on 7-OHCP production. ATP and glucose-6-phosphate omissions decreased the yields by 3% to 6%. Nicotinamide and NADP were the most vitally needed co-factors. While 4 μ mol of NADP per g of liver yielded up to 41.5% 7-OHCP (Table I), decreasing this co-factor resulted in proportionately lower yields. Thus, 0.8 μ mol NADP, or 20% of the normally applied quantity, lowered the yield of 7-OHCP by approximately 30%. Total omission of nicotinamide resulted in the loss of 80% to 90% of the normal yield of 7-OHCP.

Effect of Storage of Microsomes on Drug-Metabolizing Pathways

Since one of the objects of this study was to prepare 3H -7-OHCP, we attempted to vary the conditions of storage of the hepatic microsomes to eliminate some metabolic pathways in favor of 7-hydroxylation. Tables II and III summarize our results in this respect. Table II represents the results

on different storage conditions for the microsomes from a single male liver, M 1. The first vertical column indicates the metabolites formed by the fresh preparation, the second column after freezing for 4 days, the third column after refrigeration for 4 days, and the fourth column after freezing for 92 days and subsequent refrigeration for 3 days. It is obvious that the total metabolic potential, (expressed reciprocally by the percentage of unmetabolized CP), declined steadily, from about 98% for the fresh preparation, to 93% for 4 days of freezing, and 87% for 4 days of refrigeration. 3 Months of frozen storage, followed by 3 days of refrigeration produced an overall decline to 41%. With respect to production of the desired metabolite, however, the results were encouraging: While freezing for 4 days was neither useful nor harmful, and the various metabolites remained qualitatively and quantitatively almost unchanged from fresh conditions, refrigeration for 4 days nearly doubled the yield of 7-OHCP, and even 3 months freezing followed by 3 days of refrigeration produced more of the desired metabolite than the fresh preparation.

Table III summarizes the data for various conditions of storage for the pooled microsomes of 3 male and 1 female livers: Freezing for 54 days showed selective losses in the demethylation and sulfoxidation pathways. This eliminated some of the unwanted metabolites, and enhanced hydroxylation without simultaneous sulfoxidation, nearly fourfold. One half of the applied CP was converted to 7-OHCP under these conditions. Further refrigerated storage after the initial 54 days of freezing produced an additional increase in the yield, at the expense of other metabolites, yielding 67.5% of the desired 7-OHCP, the highest yield obtained to date. As expected, prolonged refrigeration for 28 days resulted in an overall low metabolic potential, with a poor residual yield of 6.7% 7-OHCP, representing about one half of the fresh value of 13.9%.

Thus, the hydroxylation system of sheep liver microsomes was shown to be remarkably resistant to deterioration by storage, and, on the contrary, storage manipulation could be successfully used to increase the yields of some metabolites at the expense of others.

Preparation of ³H-7-Hydroxychlorpromazine

For preparative purposes and characterization by mass spectrometry, 100 to 250 µl of the original 500 µl ethyl acetate solution of the CP metabolites produced by the microsomal incubation were applied in a single spot, approximately 3 cm from either side of the glass plate coated with 1 mm silica gel H. The plate was then run in a two-dimensional process, using freshly distilled solvents, first in diethylamine and subsequently in acetone in the second direction. Many solvent systems were tried to achieve optimum separation of the phenolic and the non-phenolic metabolite spots, singly and in combination. The selection of the system adopted afforded optimum conditions for the separation of 7-OHCP, regardless of any other metabolite. There was no overlap or very close proximity of any other metabolite spot. The nearest compound, seen in some instances, was an unidentified blue spot, with the color characteristics of the 8-hydroxychlorpromazine derivatives. It was, however, neither 8-hydroxychlorpromazine nor its sulfoxide, but may well have been a demethylated derivative of either. As this spot was not seen in all instances and was a minor one, when present, no attempt was made to further identify the compound.

The two-dimensional solvent system diethylamine/acetone must be applied in the sequence indicated, although this involves the inconvenience of removing adhering diethylamine before using acetone in the second direction. After the diethylamine run, the plate is kept in an evacuated chromatography oven for 40 hours at room temperature in the dark, and for an additional

8 hours at 40°C. When no further amine odor was detected, nitrogen was applied in a gentle stream to remove traces of solvent. The plate is then run in the second direction in acetone. Both runs require 50 to 60 minutes each. The Rf of the final location of 7-OHCP is approximately 0.5-0.6 in the diethylamine direction, and more variable in the acetone direction, depending apparently on traces of diethylamine adhering to the plate. The approximate Rf's varied from 0.1 to 0.3, with the latter representing the approximate average value for the acetone direction.

For characterization of the 7-OHCP, the plates were not sprayed, but viewed under a Blak-Ray UV light. The 7-OHCP spot--the major spot--appears weakly fluorescent, with a bluish to purple tinge. Other metabolites appear as more yellowish, and weakly fluorescent spots, readily distinguished from 7-OHCP. Moreover, even short exposures to UV will produce some free radical formation in the 7-OHCP spot, manifested by appearance of some color in the unsprayed spot (Forrest, Kosek et al., 1970).

To elute the 7-OHCP spot, the area marked under the UV light was scraped off, the silica powder transferred to a 13 ml screw top centrifuge tube, and shaken with 7 ml methanol for 10 minutes, after replacing the head space air with nitrogen. The tube was then centrifuged for 5 minutes at 300 x g. This procedure was repeated five times, and the pooled methanol extracts taken to dryness on a rotary evaporator. The residue was used for various further procedures. Aliquots served in the determination of elution efficiency by radioquantitation, which exceeded 80%. Thus, in a microsomal incubation in which 50% of applied ³H-CP were converted to 7-OHCP and used for thin layer chromatography, approximately 40% of the applied chlorpromazine was obtained as yield of 7-OHCP.

Other aliquots served for identification of the compound by thin layer chromatography in one-dimensional solvent systems (Forrest, Bolt and Serra, 1968) and yielded single spots of characteristic Rf. 7-OHCP was further identified by its spectrophotometric characteristics, using the procedure reported for maximum color development in 7-OHCP. The shape of the recorded curve, with peak absorption at 565 nm, was identical to that of reference 7-OHCP (Forrest, Bolt and Serra, 1968).

Various samples of 7-OHCP derived in repeated microsomal incubations were subjected to mass spectrometry using a Finnegan 1015 low resolution quadrupole mass spectrometer. It was noted that by the criteria of mass spectrometry, the prepared ^3H -7-OHCP was not as pure as the cold reference metabolite supplied by the National Institute of Mental Health.

Although only tracer amounts of the ^3H -label were present in the 7-OHCP preparation, they were considered the potential source of the greater quantities of degradation products. Radioquantitation of a thin layer chromatogram of a methanol solution of solid label showed 94.7% pure ^3H -CP, 0.9% promazine, 0.2% promazine sulfoxide, 0.4% at the origin (presumably in form of a dimer or other low polymer), and 3.8% of the radioactivity of the residual support, comprising unknown derivatives. Freshly prepared free base of the label was subjected to mass spectrometry, and showed small amounts of contamination with promazine derivatives, comparable to those seen by thin layer chromatography. An aqueous solution of the label, which had been kept refrigerated for about 45 days, and was subjected to mass spectrometry in form of the free base, however, showed more significant breakdown. Promazine and its sulfoxide were again seen in addition to CP. Under the conditions of storage, it appears that the well known phenomenon of elimination of the chlorine

from the nucleus by free radical reactions accounts for the formation of promazine derivatives. The same observation was made by Beckett, Heaven and Robinson (1963) and by ourselves in 1963, when aqueous CP solutions were aerobically exposed to UV irradiation. The presence of the ^3H -label seems to promote this type of reaction even in the solid state, and in prolonged storage of the aqueous solution, without undue exposure to light and air. Mass spectrometry of solid unlabeled CP or its aqueous solution, stored in the refrigerator for 3 months, showed no comparable contamination with breakdown products.

The major fragments in the mass spectra of CP, 7-OHCP and their respective sulfoxides have been summarized in Table IV. Additional data on the mass spectrometry of phenothiazine derivatives have previously been reported (Duffield, Craig and Kray, 1968; Audier, Azzaro et al., 1968; Guedj, Cambon et al., 1968; Audier, Cambon et al., 1968; Gilbert and Millard, 1969; Heiss and Zeller, 1969).

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RÉSUMÉ

Le Métabolisme de la Chlorpromazine dans le Mouton

II. Métabolisme in vitro et Préparation de la ^3H -7-Hydroxychlorpromazine

L. G. Brookes, M. A. Holmes, I. S. Forrest

V. A. Bacon, A. M. Duffield & M. D. Solomon

Les microsomes hépatiques de mouton incubés avec la chlorpromazine produisaient les métabolites normalement formé par cette route. Leur capacité d'hydroxylation à la position 7 du noyau étaient toute fois remarquable. Les préparations de microsomes fraîches convertaient d'un tiers jusqu' à plus de deux tiers du substrat en dérivés divers de la 7-hydroxychlorpromazine. La manipulation des conditions de préservation de la fraction microsomale précédant l'incubation avec la chlorpromazine, rendait presque 70% de 7-hydroxychlorpromazine pure au frais d'autres métabolites phénoliques et non-phénoliques. Donc, dans les microsomes hépatiques du mouton, les voies métaboliques d'hydroxylation à la position 7 résistaient mieux à la détérioration en préservation au froid que celles de déméthylation et d'oxidation aux groupes S et N.

ZUSAMMENFASSUNG

Chlorpromazin Metabolismus in Schafen

II. In vitro Metabolismus und Darstellung von ^3H -7-Hydroxychlorpromazin

L. G. Brookes, M. A. Holmes, I. S. Forrest

V. A. Bacon, A. M. Duffield & M. D. Solomon

Inkubation von Schaflebermikrosomen mit dem Substrat Chlorpromazin lieferte die üblichen bekannten Metaboliten. Die Kapazität für 7-Hydroxylierung war bemerkenswert. Frische Präparate von Mikrosomen transformierten ein Drittel bis mehr als zwei Drittel des Substrates zu verschiedenen

7-hydroxylierten Metaboliten. Manipulation der Aufbewahrungsbedingungen der Mikrosomenpräparate vor der Inkubation mit dem Substrat ergab eine Ausbeute von nahezu 70% an reinem 7-Hydroxychlorpromazin, auf Kosten der anderen phenolischen und nicht-phenolischen Metaboliten. In Schaflebermikrosomen war daher das Enzymsystem für 7-Hydroxylierung beständiger als die Systeme für Demethylierung und S- und N-Oxidation.

TABLE I

In Vitro ³H-Chlorpromazine Metabolism by Fresh Sheep Liver Microsomes

	I Pool (2 livers ^a)	II Pool (2 livers ^a)	III M 1 ^b	IV M 2 ^b	V M 3 ^b	VI F ^b	VII Pool (III-VI)
CP, unmetabolized	30.7	41.0	1.7	1.7	2.6	1.2	1.2
nor ₁ CP	4.8	4.1	2.9	1.4	2.8	1.2	1.5
nor ₂ CP	2.3	-					
CPNO	}17.9	}5.1	10.3	12.9	24.3	11.0	11.8
CPONO			5.7	5.6	9.2	9.2	6.9
CPO	5.9	6.3	5.2	2.0	5.1	2.2	2.5
nor ₁ CPO			3.1	1.3	2.1	2.0	2.5
7-OHCP	34.0	41.5	16.4	12.9	7.8	14.0	13.9
nor ₁ 7-OHCP	2.8		9.7	5.2	4.8	3.6	4.1
7-OHCPO			}42.4 ^c	}54.7 ^c	}37.7 ^c	}52.7 ^c	}52.8 ^c
nor ₁ 7-OHCPO							
unidentified, non-phenolic	0.4		1.0	0.8	1.0	0.8	1.0
residual			1.5	1.7	2.4	2.2	1.8
total metabolized	68.1	57.0	98.4	98.5	97.4	97.9	98.2
total accounted for	(98.8)	(98.0)	(99.9)	(100.2)	(99.8)	(100.1)	(100.0)

^a unidentified breed, unspecified sex^b unidentified breed; M = male, F = female^c contains 7-OHCPO, nor₁7-OHCPO, plus a small amount of an unidentified phenolic metabolite (derived from 8-OHCP?)

TABLE II
Effects of Storage of Microsomes on ³H-Chlorpromazine Metabolite Formation
(Single liver, male, M 1)

	fresh	frozen 4 days	refrigerated 4 days	frozen 92 days, then refrigerated 3 days
CP, unmetabolized	1.7	6.6	13.3	59.2
nor ₁ CP	2.9	3.3	9.4	2.3
CPNO	10.3	21.7	14.5	
CPONO	5.7	6.6	2.6	
CPO	5.2	4.7	9.1	8.3
nor ₁ CPO	3.1	1.9	2.8	
7-OHCP	16.4	14.8	31.9	19.1
nor ₁ 7-OHCP	9.7	3.6	4.9	0.9
7-OHCPO	}42.4*	}33.7*	}7.2*	2.2
nor ₁ 7-OHCPO				
unidentified deaminated SO derivative, non- phenolic	1.0	1.3	1.5	1.4
residual	1.5	1.8	2.8	1.8
at origin, unidentified				2.2
phenolic, unidentified deaminated				1.5
unidentified phenolic (blue when sprayed)				1.1
total accounted for	(99.9)	(100.0)	(100.0)	(100.0)

* contains 7-OHCPO, nor₁7-OHCPO, plus a small amount of an unidentified phenolic metabolite (derived from 8-OHCP?)

TABLE III

Effects of Storage of Microsomes on ^3H -Chlorpromazine Metabolites Formed
(Pool, 3 male and 1 female liver microsomes)

	fresh	frozen 54 days	frozen 54 days, then refrigera- ted 4 days	refrigerated 28 days
CP, unmetabolized	1.2	15.9	10.2	78.6
nor ₁ CP	1.5			
CPNO	11.8	8.3	2.3	trace
CPONO	6.9	3.3	1.2	
CPO	2.5		4.5	6.5
nor ₁ CPO	2.5			
7-OHCP	13.9	50.5	67.5	6.7
nor ₁ 7-OHCP	4.1	7.7	3.8	3.3
7-OHCPO	}54.6*	}8.4*	}1.8*	0.7
nor ₁ 7-OHCPO				0.9
unidentified phenolic (blue when sprayed)			2.6	
at origin, unidentified				1.1
unidentified non-phenolic SO derivative		3.7	5.0	0.5
residual	1.8	2.2	0.8	1.6
total accounted for	(100.0)	(100.0)	(99.7)	(99.9)

* contains 7-OHCPO, nor₁7-OHCPO, plus a small amount of an unidentified phenolic metabolite (derived from 8-OHCP?)

TABLE IV

Major Fragments in the Mass Spectra of Chlorpromazine Derivatives

CHLORPROMAZINE		CHLORPROMAZINE SULFOXIDE		7-HYDROXY CHLORPROMAZINE		7-HYDROXY CHLORPROMAZINE SULFOXIDE	
m/e	%	m/e	%	m/e	%	m/e	%
318/320	19	334/336	3	334/336	10	350/352	3
272/274	12	317/319	5	289/291	4	334/336	3
232/234	5	289/291	3	288/290	3	333/335	2
86	28	272/274	4	248/250	5	305/307	2
58	100	246/248	41	86	26	288/290	3
		233/235	6	58	100	262/264	30
		232/234	7			248/250	6
		214/216	10			230/232	6
		86	3			86	8
		85	2			85	4
		84	13			84	13
		58	100			58	100